Sequence Variations of MicroRNAs in Human Cancer: Alterations in Predicted Secondary Structure Do Not Affect Processing

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Abstract

Expression levels of microRNAs (miRNAs) are globally reduced in cancer compared with matched normal tissues, and miRNA function has recently been implicated in tumorigenesis. To test whether epigenetic silencing contributes to miRNA suppression in tumors, lung cancer cells were treated with inhibitors of DNA methylation or histone deacetylation. No significant alteration in miRNA expression was detected using microarray profiling. To search for tumor-associated mutations that could affect processing and expression of mature miRNAs, a panel of 91 cancer-derived cell lines was analyzed for sequence variations in 15 miRNAs implicated in tumorigenesis by virtue of their known target transcripts (let-7 family targeting oncogenic Ras) or their localization to sites of frequent chromosomal instability (miR-143, miR-145, miR-26a-1, and miR-21). No mutations were detected within any of the short mature miRNA sequences. In addition to previously reported polymorphisms, 1 sequence variant in a precursor miRNA and 15 variants in primary miRNA (pri-miRNA) transcripts were identified. Despite pri-miRNAs having dramatic changes in the predicted secondary folding structure flanking putative cleavage sites, processing and miRNA maturation were not affected *in vivo*. Thus, genetic variants in miRNA precursors are common in cancer cells but are unlikely to have physiologic significance. (Cancer Res 2006; 66(12): 6097-104)

Introduction

MicroRNAs (miRNAs) comprise a class of small noncoding RNA oligonucleotides that are processed from larger transcripts and regulate expression of target transcripts through hybridization to their 3′-untranslated sequence, leading to either suppression of mRNA translation or induction of mRNA cleavage via RNA interference (1). *miRNA* genes may be embedded within intronic regions of coding genes and coregulated with the larger transcript or may be localized outside of known transcriptional units and subject to independent transcriptional regulation. The primary miRNA (pri-miRNA) transcript is folded into a complex secondary structure, which is cleaved to a precursor miRNA (pre-miRNA) by the nuclear RNase Drosha (2). The pre-miRNA is transported into the cytoplasm by exportin-5 (3) and further processed into the mature 17 to 25 nucleotide miRNA by the cytoplasmic RNase Dicer (4–6). miRNAs are thought to target a broad range of coding transcripts (7), with a complex level of redundancy. *In silico* target predictions have revealed that highly related or identical, geographically clustered miRNAs may target a single mRNA, whereas distinct miRNAs may also target different sequences within the 3′-untranslated region (UTR) of the same mRNA (8–10). However, with few exceptions, physiologic targets for miRNAs remain to be identified, and the fact that mature miRNAs are short and typically contain several sequence mismatches with their target transcripts has complicated computational target predictions.

Although the apparent level of redundancy in miRNA-mediated regulation would suggest that a single dysregulation or mutation in a cancer cell would be insufficient to result in a biological effect, several recent observations have pointed to altered miRNA regulation in human cancer (11–13). Microarray-based measurements of miRNA levels in a large number of primary tumors and derived cell lines have shown that global expression levels of these noncoding RNAs are significantly reduced when compared with matched normal tissues (11, 14, 15). The molecular mechanism of this down-regulation is unknown; hypothetically, specific transcriptional inhibition, epigenetic mechanisms of DNA methylation and histone deacetylation, mutations affecting processing and maturation, or regulation of miRNA stability could cause this decrease. Within this lower expression window in cancer cells, the relative pattern of specific miRNA expression seems to be correlated with tumor biology and clinical prognosis (15, 16). Biological information linking specific miRNAs to cancer has also been derived from initial studies in model organisms. For instance, the let-7 family of miRNAs regulates Ras-dependent developmental pathways in *Caenorhabditis elegans* (17), and human let-7 has recently been found to target Ras transcripts in mammalian cells as well (14). Several *let-7* genes are localized to regions of chromosomal instability that are deleted in human cancer (18), and clinical studies in lung cancer have suggested that lower levels of let-7 expression may be correlated with a shortened survival, whereas its ectopic expression suppresses colony formation *in vitro* (15). Another cluster of miRNAs localized to a locus that is targeted by deletions in chronic lymphocytic leukemia (CLL), miR-15a, and miR-16-1, has recently been implicated in both sporadic cases of CLL (19) and inherited predisposition to this leukemia (16). One specific germ-line mutation in the primary transcript of these miRNAs may affect expression of both precursor and mature miRNA (16, 20).

We report here that sequence variations in miRNA precursors are common in cell lines derived from diverse epithelial cancers but that the physiologic significance of these changes must be interpreted with caution. Screening 15 miRNAs linked to tumorigenesis by virtue of their mRNA targets or their chromosomal localization in 91 cancer-derived cell lines, we could find no evidence of mutations that altered the sequence of the mature miRNA. We identified 16 sequence aberrations in miRNA precursors, including some with effect on their secondary structure, but none altered the ability of precursors to be processed to the mature form *in vivo*.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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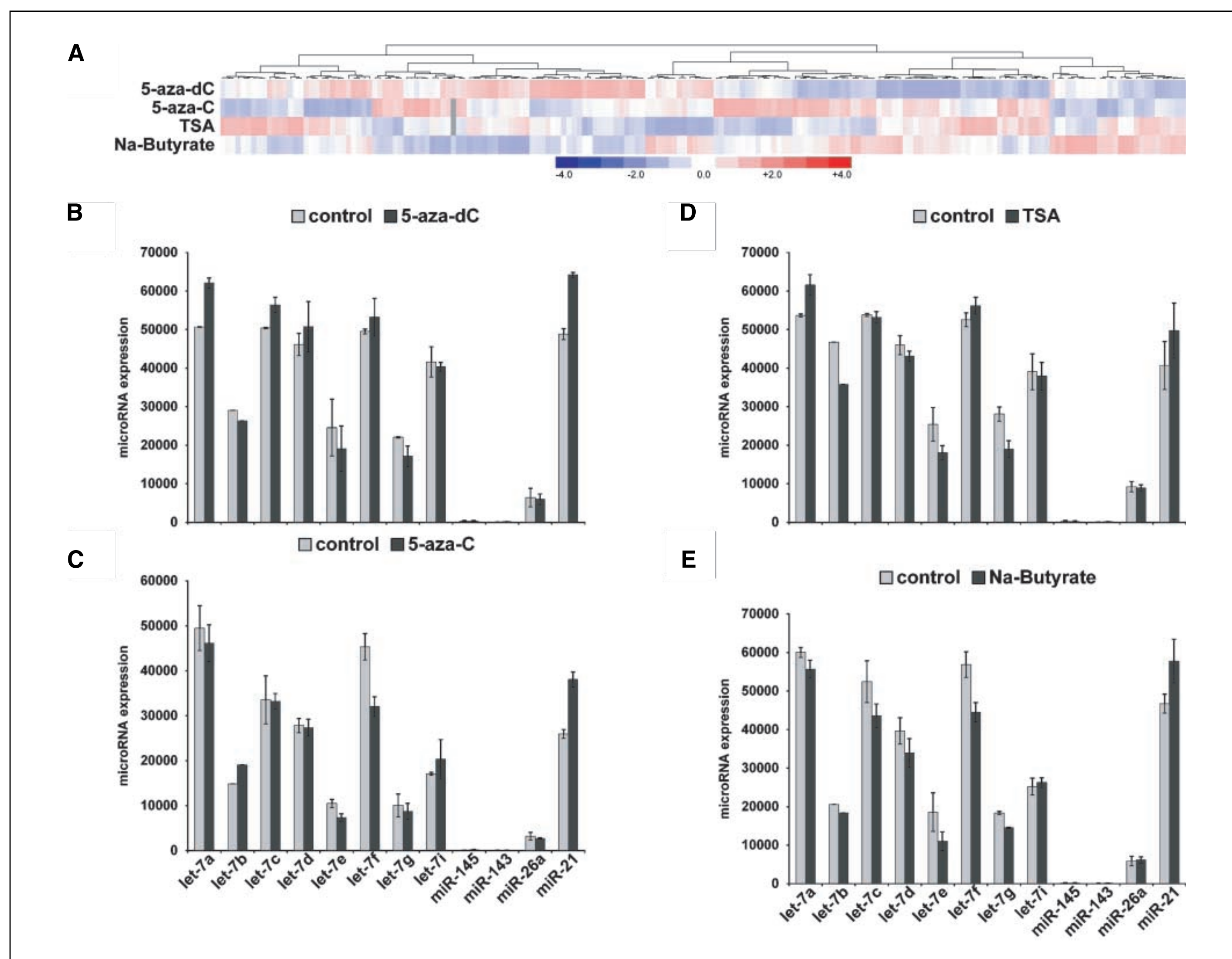

Figure 1. Epigenetic regulation of miRNA expression. *A,* A549 lung cancer cells were treated with either demethylating agents (5-aza-dC and 5-aza-C) or HDAC inhibitors [TSA and sodium butyrate (*Na-Butyrate*)] to release epigenetic silencing by DNA methylation or histone deacetylation. miRNA fraction was isolated, and the miRNA expression profile was analyzed by microarray. Cluster analysis of all miRNAs analyzed. *Red,* induction; *blue,* reduction of miRNA expression (log-scaled data). None of the analyzed miRNAs was consistently and significantly induced by DNA demethylation or HDAC inhibition. *B* to *E,* 11 mature miRNAs linked previously to cancer were selected for further analyses. Neither the Ras-regulatory let-7 miRNAs nor other miRNAs described previously to be reduced in cancer cell lines were significantly induced by treatment with either demethylating agents (*B*, 5-aza-dC; *C*, 5-aza-C) or HDAC inhibitors (*D*, TSA; *E*, sodium butyrate).

Materials and Methods

Profiling for epigenetic regulation of miRNA expression. A549 lung cancer cells were treated with the demethylating agents, 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC) or 2 μmol/L 5-azacytidine (5-aza-C), for 72 hours or with the histone deacetylase (HDAC) inhibitors, 0.5 μmol/L trichostatin A (TSA; all were from Sigma, St. Louis, MO) or 5 mmol/L sodium butyrate (Upstate, Lake Placid, NY) for 6 hours. These standard conditions have been described previously to induce silenced protein-coding genes in cancer cells (21). The small molecular weight RNA fraction was isolated from these cells using the mirVana isolation kit (Ambion, Austin, TX) and hybridized to commercially available miRNA microarrays (22).

miRNA sequencing. For sequencing analysis, genomic DNA spanning 15 *miRNA* genes was amplified by PCR (primer sequences and conditions in Supplementary Table S2) from a panel of 91 human tumor cell lines (listed in Supplementary Table S3) and subjected to bidirectional automated sequencing (Applied Biosystems, Foster City, CA) as described previously (23).

pri-miRNA secondary structure prediction. Secondary structures for the wild-type (WT) and variant pri-miRNA sequences amplified for sequencing were predicted using MFold 3.2 (24).[1] In all cases, the folding structures with minimal free energy are depicted. miRNA precursor hairpins as listed in the miRNA registry are colored in green.

miRNA cloning, expression, and Northern blotting. To express precursors for specific miRNAs, pri-miRNA sequences were PCR amplified from genomic DNA (primers listed in Supplementary Table S2) and cloned into pcDNA3.1D-TOPO-V5/His (Invitrogen, Carlsbad, CA). Expression constructs were transfected into either U2OS or 293 cells using LipofectAMINE 2000 according to the manufacturer's recommendations (Invitrogen). After 48 hours, total RNA was isolated using Trizol (Invitrogen), and equal amounts of RNA were subjected to PAGE followed by Northern blotting. miRNAs were detected using cDNA oligonucleotides (Supplementary Table S2), which were radioactively labeled at their 5′-end using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA).

[1] http://www.bioinfo.rpi.edu/applications/mfold/old/rna/.

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Results

Microarray analysis of miRNA expression following reversal of DNA methylation and histone deacetylation in lung cancer cells. The globally reduced levels of miRNA expression observed in cancer cells compared with their normal tissue of origin have led to the suggestion that epigenetic silencing may contribute to their down-regulation, resulting in increased expression of oncogenic transcripts targeted by these noncoding RNAs. As a first step to explore this possibility, we used a panel of pharmacologic agents to reverse DNA methylation and histone deacetylation, in combination with hybridization to microarrays representing 184 identified human miRNA sequences. A549 lung cancer cells are known to express significantly lower levels of let-7 miRNA than are present in normal lung tissue (15) and were therefore used in this analysis. Following treatment with either of two demethylating agents, 5-aza-dC or 5-aza-C, or either of two HDAC inhibitors, TSA or sodium butyrate, we compared miRNA expression levels by microarray hybridization (Fig. 1*A*). No significant alterations in miRNA expression patterns were found consistently across cells treated with either DNA methylation or histone acetylation–modifying agents (Supplementary Table S1), suggesting that global epigenetic silencing of miRNA expression is unlikely to underlie the reduced expression observed in these cancer cells.

To analyze a subset of miRNAs that might have a specific role in tumorigenesis, we identified 15 candidate *miRNA* genes based on their target mRNA transcripts or chromosomal localization. As noted above, the let-7 family of miRNAs comprises 11 members within the human genome, whose mRNA targets include the Ras oncogenes and whose expression is greatly reduced in human lung tumors and cancer cell lines (14, 15, 25). Several *let-7* genes are also located at fragile sites and genomic regions implicated in human cancer, including chromosome loci 3p21.1-21.2 [*let-7-g;* loss of heterozygosity (LOH) in lung and breast cancer (26)], 9q22.3 [*let-7a-1*, *let-7d*, and *let-7f-1*; LOH in urothelial (27) and bladder cancer (28)], 11q23-q24 [*let-7a-2*; LOH in lung (29), ovarian (30), and cervical carcinoma (31)], and 21q11.1 [*let-7c*; homozygous deletion or LOH in lung cancer (32)]. In addition to the let-7 family, the miRNAs *miR-145* and *miR-143* are localized close to each other at a site at chromosome 5q32 deleted (e.g., in myelodysplastic syndromes; ref. 33) and are down-regulated in various human tumors (25, 34). Two other miRNAs, *miR-26a-1* and *miR-21*, are localized to very small tumor-specific chromosomal aberrations,

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Table 1. Sequence variations of miRNAs in human cancer cells

miRNA	Variation	Location	Zygosity	Cell line
let-7a-1	C-19M(C/A)	pri-miRNA	Heterozygous	HCT-15 (colon)
let-7a-2	C-86Y(T/C)	pri-miRNA	Heterozygous	NCI-H841 (lung)
let-7a-3	C-48T	pri-miRNA	Homozygous	NCI-H596 (lung)
let-7b	delC-26	pri-miRNA	Homozygous	PC-3 (prostate)
	G+29S(C/G)	pri-miRNA	Heterozygous	UO-31 (kidney)
	G+54R(G/A)	pri-miRNA	Heterozygous	MOLT-4 (leukemia)
let-7c	C-81G	pri-miRNA	Homozygous	OVCAR5 (ovary)
let-7g	G-111R(G/A)	pri-miRNA	Heterozygous	SW626 (ovary)
				SR (leukemia)
let-7i	G-120S(C/G)	pri-miRNA	Heterozygous	SAOS-2 (bone)
				MDA-MB-231 (breast)
miR-145	C-133A	pri-miRNA	Homozygous	OVCAR8 (ovary)
	G-5R(G/A)	pri-miRNA	Heterozygous	NCI-H727 (lung)
miR-143	G-91A	pri-miRNA	Homozygous	NCI-H2291 (lung)
				SW626 (ovary)
				BT549 (breast)
				SW620 (colon)
	G-91R(G/A)	pri-miRNA	Heterozygous	SK-N-DZ (neuroblastoma)
				K562 (leukemia)
				MOLT-4 (leukemia)
				LOX-IMVI (skin)
				SK-MEL2 (skin)
				UACC-62 (skin)
				BT474 (breast)
				O28 (head & neck)
				O12 (head & neck)
miR-26a-1	G70R(G/A)	pre-miRNA	Heterozygous	ES-2 (ovary)
	A+116G	pri-miRNA	Homozygous	LOX-IMVI (skin)
	G+271A	pri-miRNA	Homozygous	O13 (head & neck)
miR-21	A+29R(G/A)	pri-miRNA	Heterozygous	HCT-15 (colon)

NOTE: Fifteen miRNAs were selected for sequencing based on their previous implications in tumorigenesis. In a panel of 91 human cancer cell lines, we found sequence aberrations in 11 of 15 analyzed miRNA sequences that had not been described as polymorphisms before. In total, we identified 16 sequence changes in pri-miRNA and pre-miRNA sequences. Nine variations were heterozygous, 6 were homozygous, and 1 occurred homozygously or heterozygously in different cell lines. Three of the sequence aberrations were recurrent.

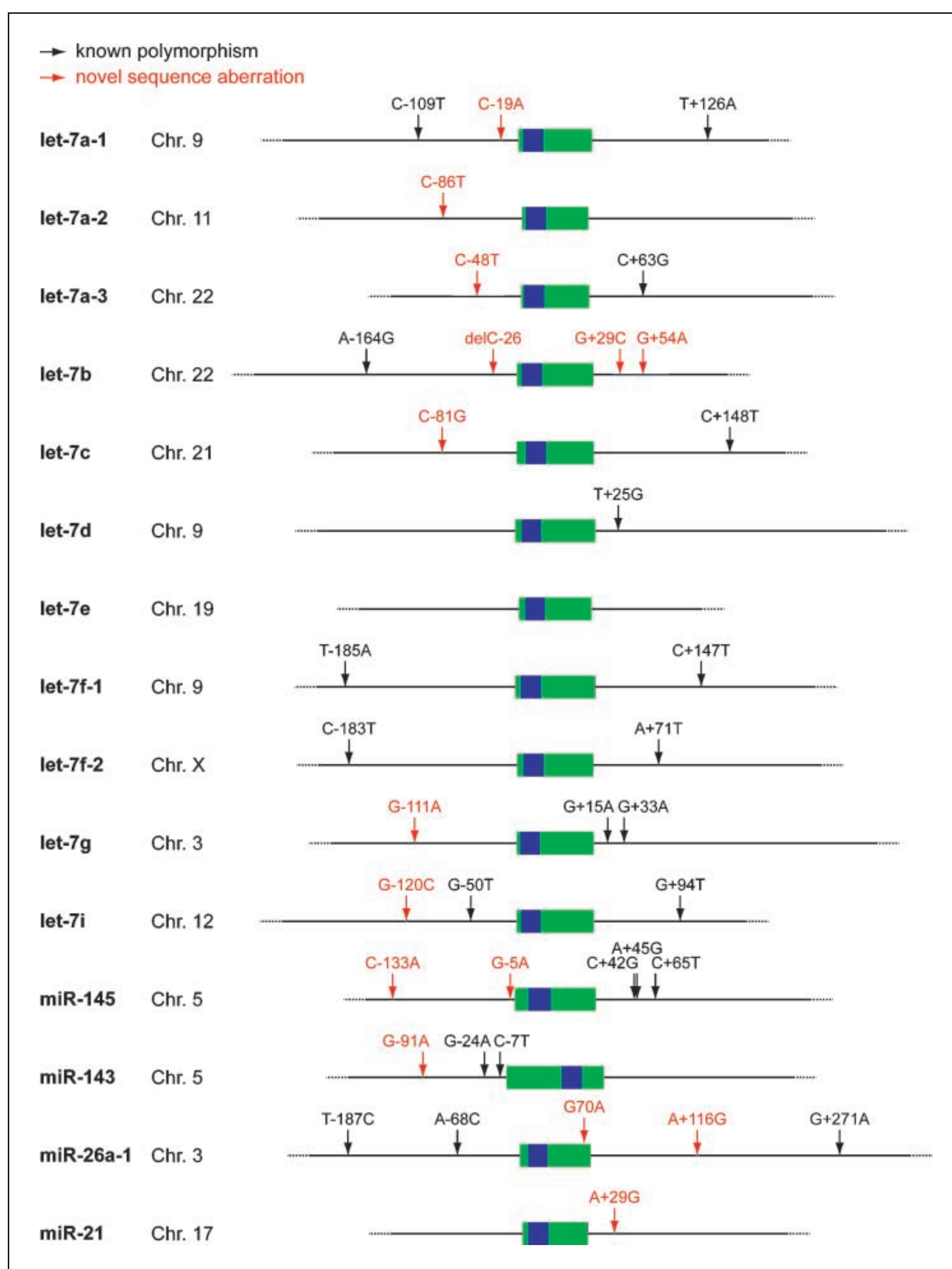

Figure 2. Localization of sequence variations in the pri-miRNA transcripts. Fifteen of the novel sequence aberrations localized to pri-miRNA transcripts (*black line*) upstream (negative base count, "−") or downstream (positive base count, "+") of the precursor hairpin (*green box*) that also contains the mature miRNA (*blue box*). *Green box,* one sequence change was detected within the pre-miRNA.

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which do not contain any known coding gene, and, hence, raise the possibility of genetic events specifically targeting these miRNAs. The *miR-26a-1* lies at chromosome locus 3p21.1, within a allelic deletion of <1 Mb reported in several epithelial cancers (35), whereas *miR-21* is localized to chromosome locus 17q23, within a small region of amplification in neuroblastoma (36). It should be emphasized that the miRNA selection for our genomic sequencing approach does not reflect a definite functional relevance of these miRNAs in tumorigenesis nor does it preclude the importance of other miRNAs in cancer because the function of most miRNAs is unknown. None of the selected potentially cancer-associated miRNAs showed significant and consistent expression differences following treatments aimed at reversing epigenetic silencing (Fig. 1*B-E*). We therefore undertook a genetic analysis of this miRNA subset using a panel of different human cancer cell lines to search for sequence-specific alterations that might point to a specific role in tumorigenesis.

Nucleotide sequencing analysis of selected miRNAs linked to tumorigenesis. The 15 *miRNA* genes described above were sequenced in a panel of 91 human cancer cell lines, representing tumors arising from lung, breast, ovary, brain, kidney, colon, skin, bone, and prostate. Genomic sequencing revealed no sequence variations within the short mature miRNAs. In addition to known polymorphisms (Human Genome Assembly National Center for Biotechnology Information 35 version 1; ref. 37; Supplementary Table S4), 1 novel variation in a miRNA precursor in pre-miR-26a-1 and 15 variations in pri-miRNAs were identified within let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7g, let-7i, miR-145, miR-143, miR-26a-1, and miR-21 in 26 cell lines (29%; Table 1). Sequence changes are numbered according to their upstream

position 5′(−) to the start of the pre-miRNA or downstream 3′(+) to the end of the pre-miRNA (Fig. 2). One of the novel sequence aberrations was homozygous or heterozygous in different cell lines, 6 were exclusively homozygous, and 9 were heterozygous. Thirteen of the 16 variations were unique, 2 were present in two cell lines, and 1 (miR-143 G-91A) was present in 13 cell lines (homozygous in 4 and heterozygous in 9; Table 1). To assess the frequency of this previously unreported sequence variation in miR-143, we analyzed a panel of control specimens. The allele *miR-143 G-91A* was identified in 13.5% of normal controls, indicating that it is a polymorphic variant.

Secondary structure alterations of variant miRNA precursors. Given the presence of novel sequence changes affecting pri-miRNA and pre-miRNA but not the mature miRNA itself, we tested whether such variants could affect their secondary structure and thereby block processing into the functional mature miRNA. We used a web-based software to calculate the most stable secondary RNA structure with the lowest free energy for each of the observed variants (24). Several sequence changes did not alter the secondary structure (e.g., delC-26 or G+54A in let-7b; Fig. 3*A*). However, some structures underwent major conformational changes even reaching into the stem of the miRNA precursor hairpin (e.g., G+29C in let-7b; Fig. 3*A*) or close to the stem reaching into the pre-miRNA (e.g., C-81G in let-7c; Fig. 3*B* and A+29G in miR-21; Fig. 4*B*). In one case, a distant single nucleotide alteration reorganized the entire secondary structure (G-91A in miR-143; Fig. 4*A*). All other structures are shown in the Supplementary Figs. S1-S4.

***In vivo* expression and processing of variant miRNA precursors.** Processing of miRNA precursors by the RNase Drosha seems to require the secondary hairpin structure characteristic of

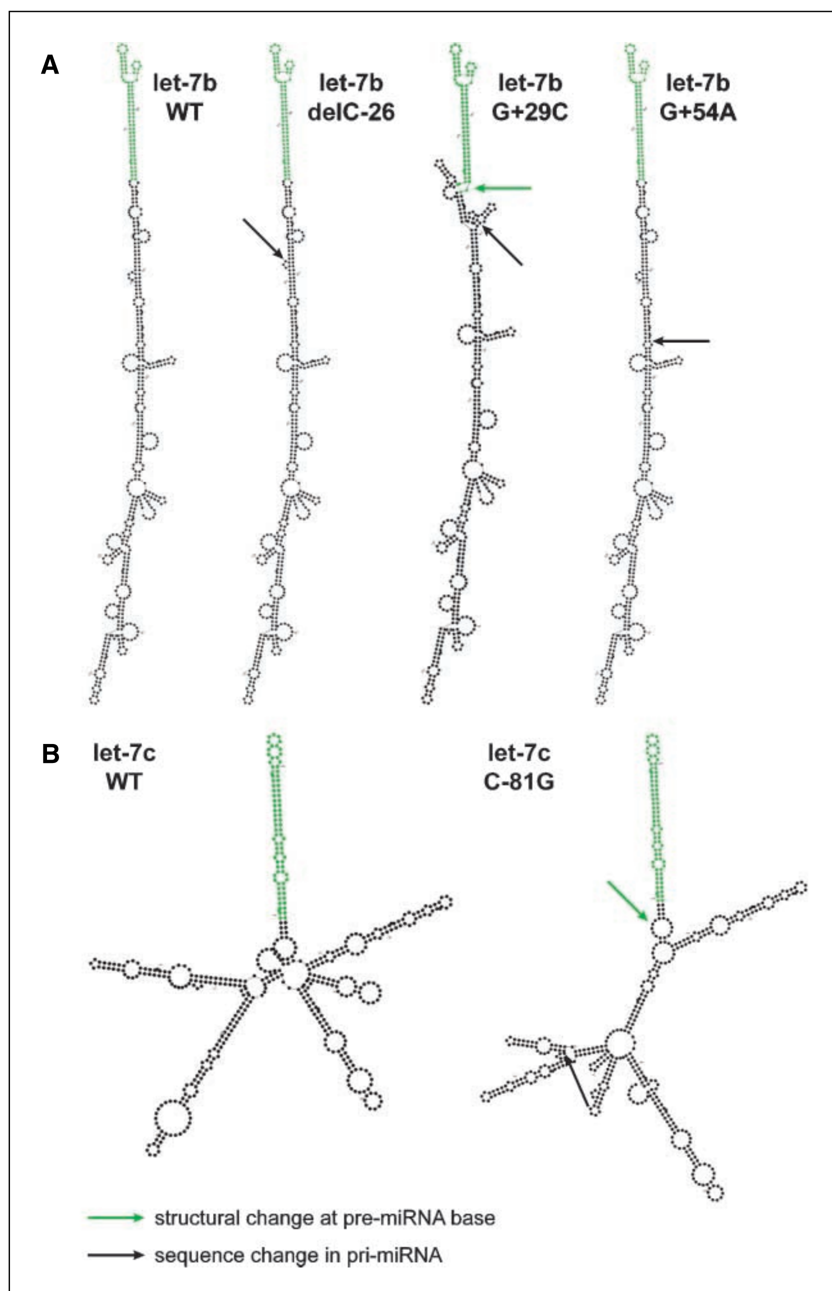

Figure 3. Sequence variations in the Ras-regulatory let-7 miRNAs can alter their secondary structure. RNA secondary structure prediction using MFold (24) revealed conformational changes in the secondary structure of the primary transcripts of the Ras-regulatory let-7 family of miRNAs that could even affect the double-stranded stem of the precursor hairpin (*green*). Depicted are only the most stable secondary structures with the lowest free energy. *A,* let-7b; *B,* let-7c.

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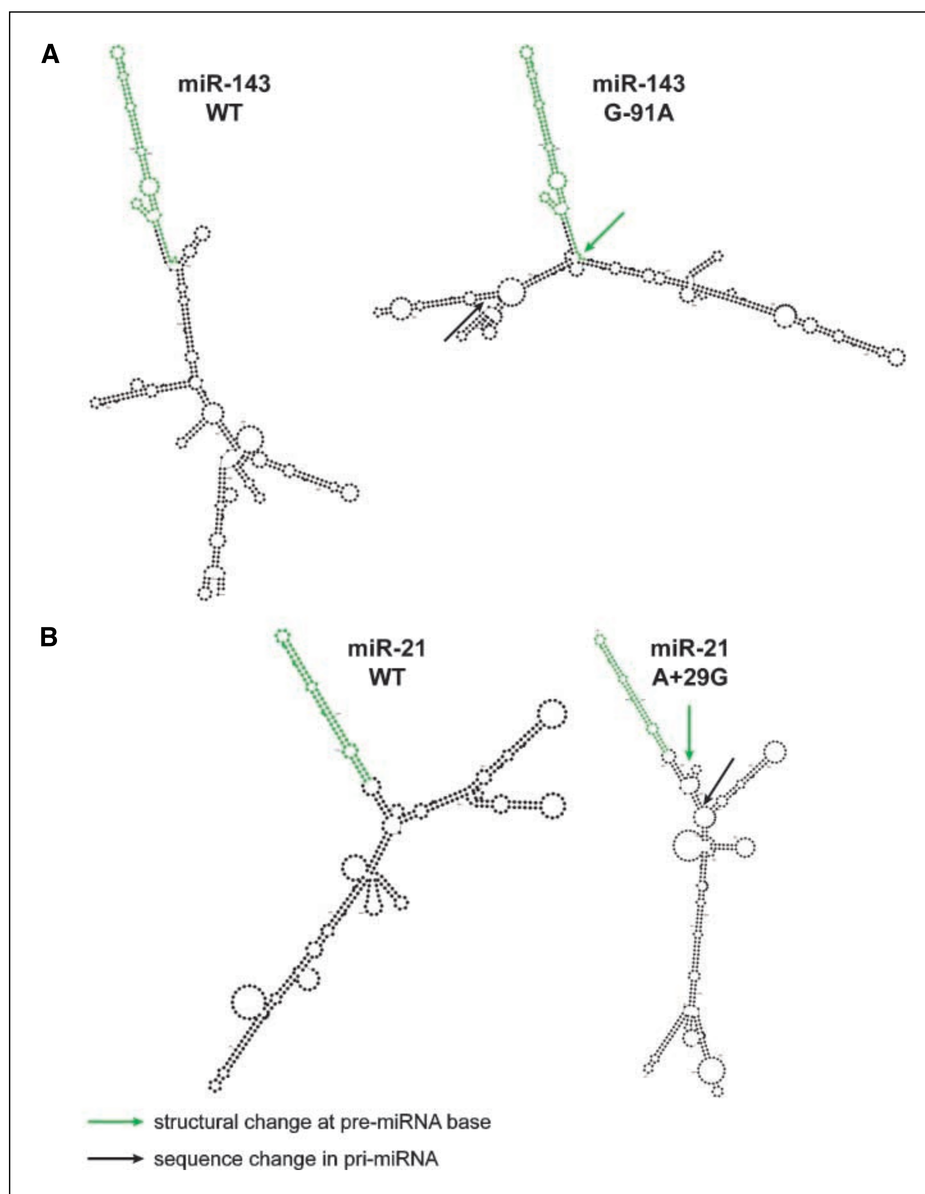

Figure 4. Sequence variations in the pri-miRNA can translate into structural alterations. RNA secondary structure was predicted using MFold (24) for WT pri-miRNAs and their novel sequence variants. Depicted are only the most stable secondary structures with the lowest free energy. *A,* miR-143; *B,* miR-21.

these RNA molecules (38), as well as specific sequence elements within the pri-miRNA (39), although these requirements have not been well characterized. To assess the effect of the predicted changes in secondary structure, we cloned expression constructs for all miRNAs and their variants and transfected these individually into either U2OS or 293 cells followed by analysis of mature miRNA expression by Northern blotting (Fig. 5). U6 snRNA and abundant small RNA species (tRNA, 5S rRNA, and 5.8S rRNA) served as loading controls. None of the sequence changes in miRNA precursors, including those with alterations in secondary structure of the precursors, led to a reduction in the expression level of the precursor itself or its cleavage into mature miRNA. Thus, despite their frequency in cancer cells, genetic variants in these miRNA precursors are unlikely to result in significant functional defects.

Discussion

The expression of miRNAs is reduced in a broad range of tumors and cancer cell lines (11, 14, 15, 25), but the mechanism underlying the general repression of miRNA expression is unknown. In our studies, miRNA expression cannot be induced by demethylating agents or HDAC inhibitors in the lung cancer cell line A549. Our observations differ from a recent report, suggesting that treatment of a breast cancer cell line with a proapoptotic dose of the HDAC inhibitor hydroxamic acid LAQ824 resulted in both up-regulation and down-regulation of many miRNA transcripts, accompanying the induction of apoptosis (40). This discrepancy may reflect different responses in the cell lines tested as well as dose-dependent effects of the different HDAC inhibitors. The lack of epigenetic regulation of miRNA transcription in lung cancer cells prompted us to analyze whether post-transcriptional steps in miRNA processing [e.g., cleavage by Drosha or Dicer, nuclear export by exportin-5, editing by adenosine deaminase acting on RNA (ADAR) or RNA stability] might be affected by sequence changes within precursor molecules.

Several lines of evidence have suggested a role for miRNAs in tumorigenesis, the most compelling being the regulation of Ras by

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the let-7 family (14, 15, 25). For our sequencing approach, we selected 15 miRNAs based on their potential link to tumorigenesis. However, because novel human miRNAs remain to be identified and the relevant targets for the vast majority of miRNAs are unknown, the selected miRNAs represent only an initial screen of sequences potentially important in cancer. Taken together, our genetic analysis of cancer cell lines representing diverse tumor types indicates relatively frequent sequence changes in pri-miRNA transcripts, less common variants in the precursor pre-miRNA, and none in the short mature miRNA. These sequence variants result in major conformational changes in the predicted secondary structure for several pri-miRNAs, but we did not observe any functional consequence on miRNA processing resulting from these aberrations.

The lack of functional significance attributable to the pri-miRNA alterations reported here differs from that reported for a single germ-line mutation in the pri-miRNA of miR-15a/miR-16-1 implicated in CLL (16). However, the potential functional defect in miR-15a/miR-16-1 processing has been questioned and needs further clarification (20). For the miRNAs studied here, the let-7 family and miR-145, miR-143, and miR-26a-1, the reported loss of expression in human cancer, cannot be attributed to genetic aberrations resulting in diminished miRNA processing. Sequence aberrations affecting miRNA processing are likely to be rare in human cancer. However, our data do not exclude the possibility that sequence variation in miRNAs and their precursors might have regulatory effects in specific cell types or dependent on the expression of specialized regulatory factors.

Our analysis indicates that single nucleotide changes at various sites, closely flanking the cleavage site or distant from the precursor hairpin, can have a profound effect on the calculated most stable secondary structure with the lowest free energy. Some of the critical elements required for correct processing of the pri-miRNAs by the RNase Drosha have been identified only *in vitro*, including presence of short single-stranded overhangs adjacent to the hairpin structure (39). The secondary structure elements that contribute to efficient pri-miRNA processing *in vivo* are currently not defined.

Several different mechanisms may be invoked to explain the apparent discrepancy between the structural changes of pri-miRNAs and the absence of effect on miRNA processing and maturation. First, it is possible that the secondary structure predicted for variant precursors is not in fact achieved due to additional factors that stabilize a less favorable conformation. In cancer cells, this conformational stabilization could render it resistant to conformational changes caused by single nucleotide changes. Alternatively, it is also possible that the processing machinery, which is capable of recognizing a large array of different precursor structures, may not be hindered by what seems to be dramatic changes in secondary structure, as long as certain key elements remain present. Given the gross changes predicted in secondary structure, very small recognition elements would presumably have to be involved. Lastly, both hypotheses could be connected: The miRNA processing machinery itself could interact with the pri-miRNA sequence through the conserved hairpin and stabilize a favorable conformation that facilitates cleavage. Differentiation between these hypothetical models will require biophysical analyses in reconstituted systems.

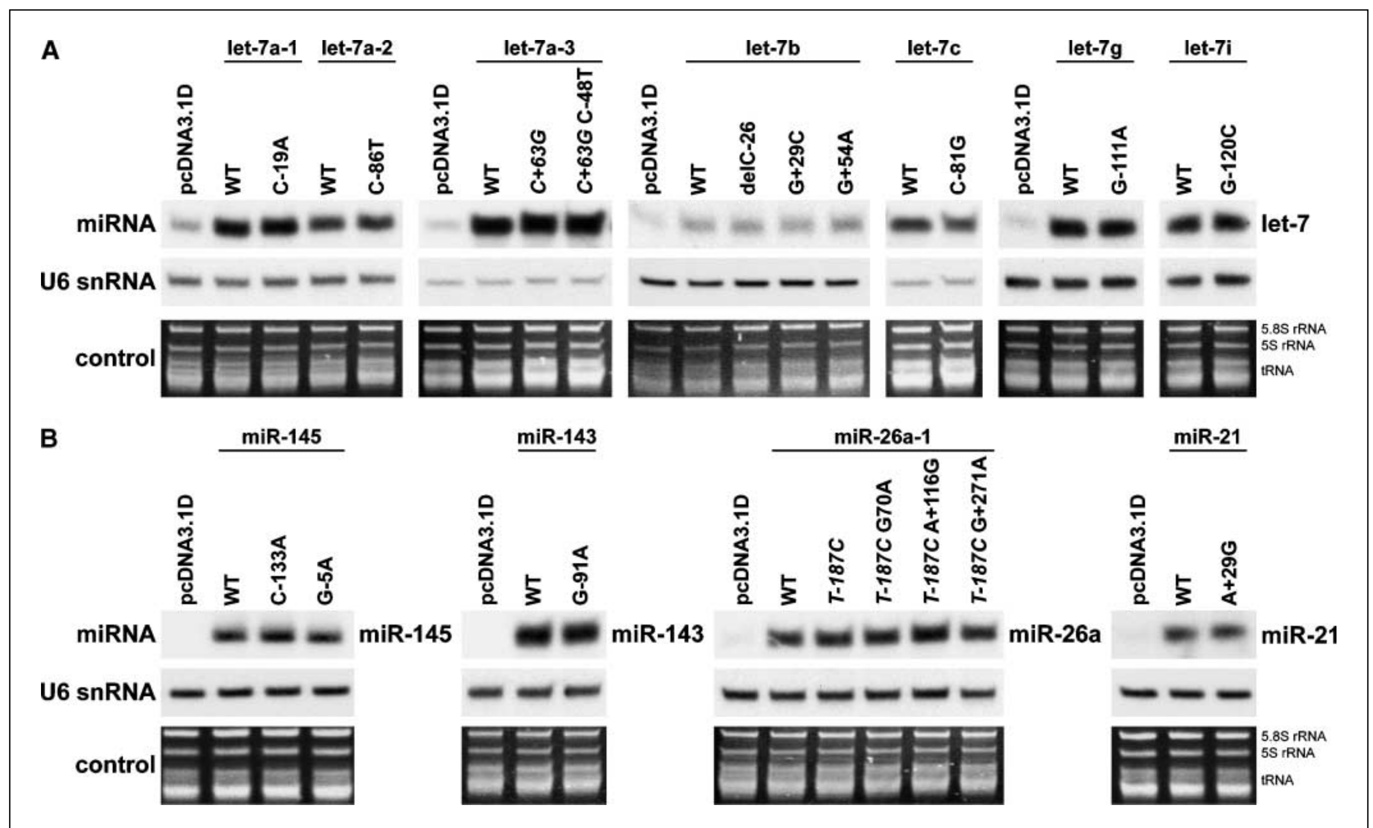

Figure 5. Sequence variations in pri-miRNA and pre-miRNA do not affect their processing. Overexpression of all miRNA sequence variants in human cells was detected by Northern blotting. *WT,* published WT sequence. Known polymorphisms are italicized. U6 snRNA (Northern blot) and abundant small RNA species (ethidium bromide staining) served as controls for equal loading. None of the sequence variations significantly impairs miRNA maturation.

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In conclusion, our data provide evidence that sequence variations of miRNAs are present in human cancer cells and that they may alter the most stable predicted secondary pri-miRNA structure but that they are unlikely to affect miRNA processing. Because individual noncoding RNA mutations are unique or rare in the population, their role in tumorigenesis cannot be readily determined by their relative prevalence in cancer specimens; hence, functional analyses are essential, much as they are for rare missense mutations in coding sequences. Despite the absence of functional mutations in the 15 cancer-associated miRNAs that we analyzed here, our data leave open the possibility of mutations in other miRNAs or post-transcriptional sequence changes induced by ADAR editing (41) as well as alterations in transcriptional regulators or promoter elements that direct miRNA expression or of mutations in miRNA-binding sites within the 3′-UTR of target transcripts. Our study highlights the importance of functional characterization of genetic variation in pri-miRNA species identified in human cancers and points to the apparent flexibility in requirements for correct processing of these noncoding RNA precursors.

Addendum

While our article was under revision, Yanaihara et al. published a study on microRNA expression in lung cancer (42). They also showed that the demethylating agent 5-aza-2′deoxy-Cytidine (5-aza-dC) and the HDAC inhibitor TSA did not alter expression of miRNAs in the lung cancer cell lines A549 and NCI-H157 supporting the absence of epigenetic regulation of miRNA expression.

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